

DIAGNOSIS OF BACTERIA AND BLOOD PARASITES

SECOND EDITION

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DIAGNOSIS OF BACTERIA
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DIAGNOSIS OF BACTERIA AND BLOOD-PARASITES

BY

E. P. MINETT

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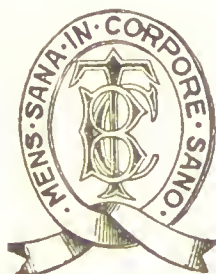
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PREFACE TO SECOND EDITION

OWING to the enormous strides made in the study of tropical diseases within the last few years, the author feels that a short summary of the more common blood-parasites should be included in a book of this description.

In order to make room for this extra summary without materially increasing the size of the book, many duplicates of staining methods have been deleted. A few well-known tests and reactions have been briefly described in order to bring the book up to date.

I beg to tender sincere thanks to Dr. H. B. Newham, of the London School of Tropical Medicine, for kindly reading the proof-sheets.

E. P. M.

GOVERNMENT PUBLIC HEALTH LABORATORY,
GEORGETOWN, BRITISH GUIANA,
February, 1913.

PREFACE TO FIRST EDITION

IN presenting this small book to students and members of the medical profession who desire a working knowledge only of a subject which is rapidly becoming of increasing importance in preventive medicine, I have endeavoured to condense the really essential points of practical bacteriology into as small a space as possible, and arranged it with a view to easy reference, in order that it may serve as a pocket laboratory companion.

I desire to express my thanks to my chief, Dr. Eyre, for his kind supervision and for reading the proof-sheets ; also to Mr. J. Turner, for his assistance in preparing cultures, films, and in many other ways.

E. P. MINETT.

BACTERIOLOGICAL LABORATORY,
GUY'S HOSPITAL, S.E.,
March, 1909.

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DIAGNOSIS OF BACTERIA AND BLOOD-PARASITES

IDENTIFICATION OF BACTERIA

MODE of procedure applied to pure culture for identification :

1. Note general appearance of culture in tube—shape, size, transparent or opaque, dry or moist, colour, etc., of colonies growing—both with naked eye and hand-lens ; also note any change of colour, liquefaction, or gas-formation in media in which colonies are growing, and kind of media used for cultivation, with a view to successful subcultivation.

2. Prepare a hanging-drop preparation with ordinary tap-water from the original culture ; observe under $\frac{1}{6}$ -inch objective with a dull field. Carefully note—

- (1) Shape of organisms, cocci, bacilli, etc.
- (2) Motility or non-motility.
- (3) Grouping—*i.e.*, clumps, pairs, chains, etc.

3. Make three cover-glass films, dry over flame in fingers (to prevent charring of specimen), and stain as under for further provisional examination :

- (1) Carbolie methylene blue.
- (2) Ziehl-Neelsen's method.
- (3) Gram's method.

4. Make subcultivations as under, and incubate for twenty-four to forty-eight hours for further investigation :

- (A) Nutrient bouillon, and incubate at 37° C. for twenty-four hours.
- (B) Nutrient agar (sloped), and incubate at 37° C. for twenty-four to forty-eight hours.
- (C) Nutrient gelatine (sloped), and incubate at 20° C. for forty-eight hours to seven days.

By means of methods A, B, and C, we can now proceed to identify the organism with one or other of the main subdivisions given under, and make a rough provisional diagnosis. The three tubes planted out and placed in the incubator can be used later to still further investigate and confirm the original culture.

In some cases special media and procedure are necessary to secure a growth. These will be mentioned as they occur under their respective organisms; also special staining processes when necessary, such as for flagella, capsules, spores, etc. ; or if an organism requires to be grown anaerobically or under any exceptional conditions as to environment, reaction of media, or temperature.

Note.—The principal staining methods are given briefly on pp. 32-42, together with a simple method for special processes, such as for flagella, capsules, spores, granules, etc. ; but as they are added merely for easy reference, a standard textbook should be consulted for description of their composition and details of methods.

The routine examination can now be proceeded with as follows, using the broth culture for the hanging-drop preparation, provided the broth is turbid on the following day, instead of the previous method, which is only intended for use when time is limited.

Hanging Drop.—Organism is a *coccus*.

Motile : *Micrococcus agilis* ; but as this organism is non-pathogenic, it is unimportant.

Non-motile : *Staphylococcus*, singly and in clumps.
Streptococcus, singly, in pairs and chains.

Pneumococcus, usually in pairs and short chains.

Tetragenus, usually in fours.

Meningococcus, *Gonococcus*, *Micrococcus catarrhalis* vary, but usually singly or in pairs.

Micrococcus melitensis, very minute.

Gram-positive cocci may be—

Staphylococcus, *streptococcus*, *pneumococcus*, or *tetragenus*. Gelatine at 22° C. Media liquefied = ***Staphylococcus***.

Note colour of colonies on the agar tube, whether aureus, albus, or citreus ; also uniform turbidity in broth, with abundant layer, settling to bottom of a yellowish-brown or white tint ; acid reaction and sour odour. Prove by—

- (1) Gelatine stab, funnel-shaped, liquefaction commencing at top second or third day, and yellow flocculent deposit.
- (2) Litmus milk, acid and firm clot.

- (3) Potato at 22° C. grows well, and growth is orange, yellow, or white in colour, according to variety of organism.
- (4) Note grouping of organisms on stained cover-slip, usually in clumps.
- (5) Best stain to use to demonstrate the organism is :
 - (a) In original pus use methylene blue.
 - (b) After cultivation, Gram's method.

No growth on gelatine ; probably **Pneumococcus**.
Prove by—

1. Blood-agar at 37° C. ; note small transparent colonies and discoloration of media to dirty brown.
2. Note small size, lanceolate shape, and arrangement in pairs on stained preparation.
3. Inoculate into mouse, and make films of heart-blood after death. Note capsules (*dark-coloured halo*), and stain for same with capsule stain.
4. Stains :
 - (1) Original specimen, carbolic methylene blue ; also, to demonstrate the capsules, the original pus or heart-blood must be used : either MacConkey's or Welch's methods give good results.
 - (2) Specimen after cultivation with Gram's stain (no capsule).

Growth poor and colonies very small, but no liquefaction of media = streptococcus.

Streptococcus, to prove :

1. Broth; numerous small discrete granules; deposit has sandy appearance, not abundant.
2. Litmus milk, acid reaction, no clotting.
3. Blood-agar at 37° C. ; minute discrete colonies having colourless halo round them in media.
4. Note length of chains of cocci in stained slides and in hanging drop from broth culture.
5. Stain :
 - (1) Original specimen, carbolic methylene blue, especially in pus.
 - (2) After cultivation, by Gram's method or weak carbol-fuchsin solution.

Abundant growth =

Micrococcus tetragenus, to prove :

1. Peptone gelatine stab: fairly thick growth along line of puncture ; thick rounded disc at surface of whitish colour.
2. Agar and potato: abundant ; moist, *slimy* layer at room-temperature, of whitish colour and very viscid.
3. Note arrangement in *fours* on stained slide, each group often surrounded by (indefinite) capsule.
4. White mice extremely susceptible ; injection subcutaneously causes general septicæmia. Prepare cover-slides from heart-blood, and observe tetrads.
5. Stain—
 - (1) Original pus: use Welch's method.
 - (2) After cultivation, carbolic methylene blue or Gram's method.

Gram-negative cocci may be—

Gonococcus, Meningococcus, Micrococcus catarrhalis, *M. melitensis*.

Gelatine at 20° C. :

- A. If growth, may be *M. catarrhalis* or *M. melitensis*.
- B. No growth may be gonococcus or meningococcus.

(A) Examine stained slide. Organism is fairly large. Cocci generally in pairs.

M. catarrhalis, to prove :

- 1. Serum agar : opaque colonies and very tough consistence.
- 2. Litmus milk : no change.
- 3. Rapid growth on gelatine at 20° C.
- 4. Stain with Gram's method, using neutral red as counter-stain.

M. melitensis = organism extremely *minute* in stained slide ; usually single cocci, but occasionally in pairs or short chains and bunches. Prove—

- 1. Pearly white colonies, with yellowish tint on agar at 37° C., turning to dark brown later on.
- 2. Extremely slow growth in gelatine at 20° C.
- 3. Moist white growth on potato.
- 4. Litmus milk : alkaline reaction.
- 5. Micrococcus can be cultivated from urine of infected animal several months after inoculation.

6. Stain : Gram's method, using weak carbol-fuchsin as counter-stain.

(B) **Gonococcus**.—Stained slide ; gonococci in pairs. Characteristic shape, like two horse-beans placed with concavities facing. Prove :

1. Very difficult to grow on ordinary media.
2. Blood-agar small, discrete, colourless colonies, like small drops of dew.
3. Smear preparation from urethral discharge ; note that nearly all organisms are intra-cellular and tend to swell up ; also, if many present in leucocyte, the nucleus is often broken up and stains badly.
4. Stain the smear preparation with carbolic methylene blue, also by Gram's method, using neutral red as counter-stain. It is most important in making a diagnosis that the coccus should be demonstrated as Gram-negative.

Meningococcus = cover-slide ; very much smaller cocci in pairs with flattened sides. Prove :

1. Blood-agar : much more luxuriant growth than gonococcus. Colonies much larger, and smooth circular discs, with smooth edges.
2. Ordinary agar growth : fairly abundant.
3. Stain—
 - (1) Original specimen with carbol-thionin blue.
 - (2) After cultivation, with Gram's method, use neutral red or weak carbol-fuchsin as counter-stain.

Fermentation tests with sugars. Gram-negative cocci:

			Glucose.	Galactose.	Maltose.
Gonococcus	+	+	-
Meningococcus	+	+	+
M. catarrhalis	-	-	-
M. melitensis	+	+	-

For non-pathogenic cocci, with distinguishing tests, see list at end of Pathogenic Bacilli.

BACILLI

Examination of Hanging-Drop Preparation.—

If organism is **motile**, it is probably—

1. *B. aerogenes capsulatus*.
2. *B. tetani*, *Vibrio cholera*, *B. pyocyaneus*,
B. typhosus, *B. coli communis*, *B. Gaertner*,
B. paratyphosus (A or B).
3. *B. enteritidis sporogenes* and Morgan's No. 1
bacillus.

Prepare Gram Cover-slide.

Gram positive = *B. aerogenes capsulatus* (probably identical with *B. enteritidis sporogenes*), *B. tetani*, *B. botulinus*.

Gelatine shake culture.

Growth with gas-formation (*i.e.*, anaerobic growth)
= *B. aerogenes capsulatus*.

No growth on surface either agar or gelatine =
B. tetani.

B. aerogenes capsulatus, to prove :

1. Gram slide—large square-ended Gram-positive organism. Usually shows well-marked capsule.
2. Gelatine at 22° C., shake preparation ; the formation of *gas* in shallow parts of media serves to distinguish from *B. tetani*.

3. Inject culture intravenously into a rabbit ; kill the animal ; incubate the cadaver six hours at 37° C. ; note animal swells up ; gas formed in all tissues, also spore-formation of organism.
4. Best stain, methylene blue or methyl violet.

Note that the organism is often non-motile, and sometimes occurs in pairs, chains, or filamentous forms, and may resemble *B. anthrax*, but is distinguished from it by being anaerobic and forming gas.

B. enteritidis sporogenes, to prove :

1. Cover-slide, shows rather large bacillus. Gram positive and showing spores, either central or nearer one end.
2. Slightly motile ; small number terminal flagella ; demonstrate by staining for flagella with special stain.
3. Inoculate 20 c.c. sterile litmus milk with culture, heat for ten minutes at 80° C. to destroy all vegetative forms of bacteria, cool tube, incubate in Buchner's tubes at 37° C. for twenty-four to thirty-six hours. Note the characteristic broken-up clot, complete separation into curds and whey, and abundant gas-formation. Smells of butyric acid. Litmus milk clot looks like broken-up red rubber.
4. Whey crowded with bacilli, and can be used for guinea-pig inoculation if required.
5. Stain methyl violet.

B. tetani, to prove :

1. No growth at 20° C. (aerobically). On glucose agar grows anaerobically at 20° C.
2. Broth or agar cultivations at 37° C. in Buchner's tubes, after subjecting the original emulsion or broth cultivation to 80° C. for half an hour to destroy vegetative forms, give good growth.
3. Cover-slide preparation shows large Gram-positive bacillus with characteristic *terminal* spores (drumstick).
4. Cultivate glucose bouillon anaerobically at 37° C., and stain to demonstrate presence of flagella.
5. Grow a culture on glucose formate gelatine for further investigation.
6. Stain :
 - (a) In tissues carbolic methylene blue.
 - (b) Cultures Gram's method.
 - (c) Broth culture with Flagella stain.

B. botulinus, to prove :

1. Cover-slide large bacillus with rounded ends. Gram positive. Note the flagella are few in number—four to eight—and are all *lateral* in position; spores, if present, are mostly *terminal* in position.
2. Glucose gelatine plates, cultivated anaerobically at 20° C. for forty-eight hours, show small spheres of liquefaction, with granules of yellowish-brown colour present. These show a constant streaming movement,

- especially at periphery. Note rancid odour of cultures, due to formation of butyric acid.
3. Cultures on glucose agar at 20° C. in Buchner's tubes. Note the abundant formation of gas and splitting up of media.
 4. Litmus milk, no coagulation.
 5. Ferments glucose, but not lactose or saccharose.
 6. Very feebly motile.
 7. Stain in carbolic methylene blue or methyl-violet.

Motile Bacilli.

Gram negative = *Vibrio cholera*, *B. pyocyaneus*, *B. typhosus*, *B. coli communis*, *B. Gaertner*, *B. paratyphosus*, Morgan's No. 1 bacillus.

A. Gelatine culture is liquefied = **V. cholera** or **B. pyocyaneus**.

V. cholera, to prove :

1. To bouillon cultivation add a few drops of pure H_2SO_4 , and note pink coloration (cholera-red reaction).
2. Add to peptone water or broth culture a few drops of a solution of paradimethyl-amine-benzaldehyde and some solution of acid sodium sulphate, and note cholera-red reaction, due to indol.
3. Cultivate on blood-agar at 37° C. for forty-eight hours, and note absence of hæmolysis.
4. Cover-slide preparation. Note shape of organism; it is like a crescent in shape, but often many are joined to form a spirillum, and organism has one terminal flagellum.

5. Pfeiffer's test applied to guinea-pig is positive.
6. Stain : weak carbol-fuchsin or silver method.

Pfeiffer's Test for Cholera Vibrio.—This reaction is based on the production of an anti-cholera serum by the injection of a serum from a highly immunized animal.

A loopful (2 milligrams) of twenty-four hours old agar culture of the suspected vibrio is added to 1 c.c. of ordinary broth, containing 0.001 c.c. of anti-cholera serum. The mixture is injected intraperitoneally into a young guinea-pig of 200 to 300 grams weight.

At the end of twenty minutes a little peritoneal fluid is withdrawn by a sterile capillary pipette. Make a hanging-drop preparation from this fluid. If the organism is the true cholera vibrio, the spirilla will be observed to be broken down, and transformed into granules.

This lysogenic action is absent if the vibrio is not the true cholera organism, the vibrios remaining alive and actively mobile.

A control injection should also be performed on another guinea-pig, using ordinary serum instead of the anti-cholera serum.

The vibrios in the control experiment should remain entire and active when withdrawn after twenty minutes and one hour, and examined in a hanging drop.

Note that the following organisms closely resemble the true cholera vibrio. Their principal differences are given here, but a textbook on bacteriology should be consulted for a detailed description.

Finkler and Prior's Vibrio.—(a) Much more rapid growth on gelatine.

(b) No cholera-red reaction in peptone water cultures for twenty-four hours.

Metschnikoff's Spirillum.—(a) Marked cholera-red reaction in peptone water; twenty-four hours old cultures.

(b) Pigeons not affected by true cholera vibrio, but are rapidly killed with septicæmic symptoms after subcutaneous injection of Metschnikoff's spirillum.

Deneke's spirillum closely resembles *V. cholera* microscopically, but is usually regarded as a comparatively harmless saprophyte.

B. pyocyaneus, to prove :

1. Gelatine culture is liquefied, and shows the characteristic green colour.
2. Agar culture, twenty-four hours at 20° C. : growth on surface, and green colour diffused throughout the medium.
3. Potato at 20° C., forty-eight hours : reddish-brown growth on surface; medium shows greenish discoloration.
4. Shake up culture with chloroform; green colour (pyocyanin) is extracted: add a weak acid: colour changes to red.
5. Stain :
 - (a) Original substance carbolic methylene blue.
 - (b) Cultures, Gram counter-stained neutral red.

B. Gelatine culture is not liquefied; organism is B. typhosus, B. coli communis, B. Gaertner, B. paratyphosus, Morgan's No. 1 bacillus.

Indol reaction positive — *B. coli, B. Gaertner, Morgan's No. 1 bacillus.*

B. coli communis, to prove :

1. Gelatine shake culture at 20° C. : note gas-formation.
2. Potato at 20° C. : brown slimy growth on surface.
3. Litmus milk : acid, gas and firm clot.
4. Note sugar reactions below.
5. Prove indol reaction in ordinary broth culture. Note also formation of gas and fæcal odour in lactose bouillon.
6. Agglutination reactions microscopic and macroscopic. (For details, see Bacteriological Technique—Eyre.)
7. Cover-slide : short, stout, rounded Gram-negative bacillus ; flagella few in number, usually about eight.
8. Stain :
 - (a) Original substance pus, etc., carbolic methylene blue.
 - (b) Culture, Gram and carbol - fuchsin (weak) as counter-stain.

“Flaginac” Test for Bacillus Coli (Houston).

—Fl. . . . greenish fluorescence in neutral red broth cultures.

Ag. . . . acid and gas in lactose peptone cultures.

In. . . . indol formation in broth cultures.

Ac. . . . acid and clotting of litmus milk.

B. Gaertner, to prove :

1. Lactose litmus bouillon : *no* gas-formation.
2. Litmus milk gives alkaline reaction.
3. Cover-slide : resembles typhoid bacillus in appearance : Gram negative. Stain for flagella.

Very numerous—sixteen to twenty-four in number.

4. Agglutination reaction.
5. Sugar reactions (see below).
6. Very pathogenic. Animals hæmorrhagic diarrhœa.
7. Stain :
 - (a) Original methyl violet or carbolic methylene blue.
 - (b) Culture Gram and weak carbol-fuchsin.

Morgan's No. 1 bacillus, to prove :

1. Litmus milk gives alkaline reaction, but very slowly. (Distinguish from *B. coli*.)
2. Mannite or dextrin litmus bouillon both negative. (Distinguish from *B. Gaertner*.)
3. No production of acid on mannite, neutral-red agar plates.
4. Stain cover-slide for flagella.
5. Produces diarrhœa in rabbits fed on cultures.
6. Stain Gram and weak carbol-fuchsin.

Indol reaction negative = *B. typhosus*, *B. paratyphosus* (a and b).

Litmus milk :

1. Acid, no clot, no gas = *B. typhosus*.
2. Acid to alkaline and gas-formation = *B. paratyphosus*.

B. typhosus, to prove :

1. Hanging drop actively motile—much more so than *B. coli communis* or other allied organisms. Often shows long filamentous forms.
2. Cover - slide : bacillus is longer and more slender than *B. coli communis*. Stain for

- flagella, and note that they are very long and numerous; also note that they are attached all along sides as well as ends.
3. Stab culture in peptone gelatine at 20° C.: note growth all along line of puncture; film radiating from centre at surface.
 4. Agglutination reactions.
 5. Sugar reactions.
 6. Potato: white growth on surface at 20° C.
 7. Stain carbolic methylene blue, Gram, or weak carbol-fuchsin.

B. paratyphosus, to prove (*a* and *b*):

1. Litmus milk acid only (*a* variety), acid reaction slowly progressing to alkaline (*b* variety).
2. *a* variety is indol+; *b* variety varies. Not at all constant.
3. Agglutination reactions.
4. Very pathogenic to animals, but more septicæmic in type than *B. typhosus*.
5. Stain weak carbol-fuchsin or carbolic methylene blue.

Coli Typhoid Group.

As this group is so very important pathologically, and as the various members are so closely related, a brief method is given, to enable the student to separate out and identify its various members; but it must be distinctly understood that it does not aim at being complete, and for a more detailed account much larger textbooks must be consulted. Also, owing to want of space, the agglutination reactions—a most important method of identification—are omitted, together with the inoculation experiments

with animals, which, of course, are impossible to the ordinary student, although the technique, together with some of the commoner post-mortem lesions observed, are mentioned on p. 62.

Coli and Typhoid Group.—Rough preliminary separation.

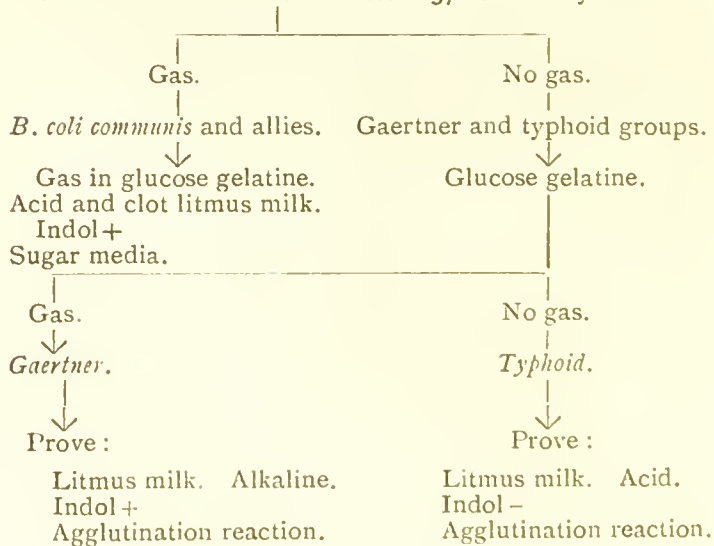
1. Smear some of the bouillon preparation showing growth on lactose litmus agar plates ; incubate for forty-eight hours at 37° C. Roughly separate out colonies as under :

Red colonies = coli group.

Blue colonies = typhoid group.

2. Lactose neutral-red agar can be used if preferred. Use the roughly separated colonies from above, cultivated on agar or in glucose bouillon, to further separate as below :

Lactose litmus bouillon. Incubate 37° C. twenty-four hours.



(Abbreviated from Eyre's "Bacteriological Technique.")

FERMENTATION, MILK, AND INDOL REACTIONS: COLI-TYPHOID GROUP.

BACILLI

19

Media.	<i>B. coli</i> <i>communis</i> .		<i>B. Gaertner</i> .		<i>B. paratyphosus</i> .		<i>B. dysenter.</i> <i>B. typhosus</i> .		<i>Morgan</i> No. 1.
	Acid.	Gas.	Acid.	Gas.	(a)	(b)			
Dextrose	+	+	+	+	+	+	+	+	+
Lævulose	+	+	+	+	+	+	+	+	+
Galactose	+	+	+	+	+	+	+	+	+
Maltose ..	+	+	+	+	+	+	+	+	?
Saccharose	o	o	o	o	o	o	o	o	o
Lactose ..	+	+	o	o	o	o	o	o	o
Raffinose	+	+	o	o	+	+	+	+	o
Dulcite ..	+	+	+	+	+	+	+	+	o
Mannite ..	+	+	+	+	+	+	+	+	o
Glycerine	+	+	+	+	+	+	+	+	+
Dextrin ..	+	+	+	+	+	+	+	+	+
Inulin ..	o	o	+	+	+	+	+	+	o
Salicin ..	o	o	o	o	+	+	+	+	o
Litmus milk	+	+	+	+	+	+	+	+	+
Indol ..	+	+	+	+	+	+	+	+	+

+ = acid reaction.
 - = alkaline.
 o = negative.
 ± = not constant.

Ehrlich's Rosindol Reaction for Detection of Indol Formation in Broth Cultures of Organisms.

Solution No. 1.

Paradimethylamidobenzaldehyde	. 4 grms.
Absolute alcohol 380 c.c.
Conc. hydrochloric acid 80 c.c.

Solution No. 2.

Potassium persulphate : Saturated solution in water.

For use : Add about 3 c.c. of Nos. 1 and 2 to about 10 c.c. of broth culture of organism.

If indol formation is present a rose-red colour slowly appears.

Bacilli.

Hanging-drop Preparation.—Non-motile : *B. diphtheriæ*, anthrax, tubercle, actinomycosis (not really a bacillus, but inserted here for convenience only), *B. pestis*, *B. dysenteriæ*, *B. Friedländer*, Influenza, Glanders, *Lactis aerogenes*.

Gram positive = *B. diphtheriæ*, anthrax, tubercle (very difficult), actinomycosis.

Gelatine at 20° C. :

1. If liquefied, probably anthrax.
2. If not liquefied = diphtheria.
3. If no growth = tubercle.
4. If characteristic button-shape colonies = actinomycosis.

B. anthrax, to prove :

1. Stroke cultivations on agar plates : Incubate at 37° C. for twenty-four hours. Examine

colonies with hand-lens or low power of microscope. Note the characteristic wavy margins, made up of long threads of bacilli.

2. Gelatine stab and incubate at 20° C. for forty-eight hours : Note the lateral spikelets radiating out into media from central stab, longest near surface and tapering downwards ; later, liquefaction commencing at surface and forming funnel.
3. Cover-slip : large square-ended bacillus, staining deeply with most stains, often in long chains, showing spores plainly. Prove presence of spores by heating to 80° C. for twenty minutes, and making further cultivations from same fluid, also see staining methods for spores.
4. Inoculation into guinea-pig subcutaneously : gelatinous infiltration at site of injection post-mortem, and prove bacillus in blood and spleen.
5. Stain :
 - (a) Original material carbolic methylene blue.
 - (b) Cultures carbolic methylene blue, Gram, and spore stains.

B. tuberculosis, to prove :

1. Cultivate on glycerine agar or on egg media at 37° C. (but rather difficult to grow).
 2. Cover-slide preparation, and prove to be an acid-fast bacillus by Ziehl-Neelsen method.
- NOTE.—For all practical purposes this test

is sufficient when time is limited, such as when examining sputum, urine, etc., but it must be remembered that other bacilli are acid-fast as well, though, with the exception of *B. lepræ*, they are all non-pathogenic. (See list of non-pathogenic acid-fast bacilli.)

3. Inoculate guinea-pig with suspected material in left popliteal region. At post-mortem note regular sequence and peculiar manner in which the glands are affected.
4. Stain, Ziehl-Neelsen.

Modified Process for Tubercular Sputum :

1. Mix 5 c.c. of sputum and 5 c.c. of 25 per cent. antiformin solution in water ; allow to stand twenty-four hours.
2. Pour off supernatant liquid, wash deposit in saline solution, collect deposit.
3. Again wash in saline solution ; allow to settle, pour off supernatant fluid.
4. Place deposit on glass slide, previously albuminized by placing a small portion of original sputum on slide.
5. Allow to dry in air, fix with heat, and stain with Ziehl-Neelsen.

NOTE.—The deposit is best separated by means of a centrifuge, but if this is not available the test can easily be carried out by sedimentation in long, narrow test-tubes.

B. Lepræ.—This organism is somewhat difficult to cultivate, necessitating special media, etc.

It is most characteristic when seen in smears from the nasal mucous membrane of a leper, or from juice expressed from a lepra nodule, or in sections of leprosy skin.

The bacilli are acid-fast, thin with pointed ends, and are usually in patches closely grouped together. They show marked plasmolysis, but no thickening in the sheath or square ends as seen in tubercle bacillus. Stain both smears and sections with Ziehl-Neelsen.

Actinomycosis, to prove (streptothrix) :

1. If in pus, observe yellow granules ; spread out some pus on thin slide ; stain with Gram, and counter-stain with weak aqueous solution of carbol-fuchsin. Observe the characteristic shapes.
 - (1) Filaments in an irregular network (Gram positive).
 - (2) Spores or gonidia (Gram positive).
 - (3) Clubs arranged round periphery of colony, and all radiating from centre (usually Gram negative). In broth cultivations note yellow pellets.
2. Glycerin agar, incubate at 37° C. for three or four days : note yellowish-red, nodular, discrete growths, very tough, and adhere firmly to media.
3. Prepare a shake culture in glucose agar, and incubate at 37° C. Note maximum growth at $\frac{1}{2}$ inch below surface of media (Wright).
4. Stain, methyl violet.

B. diphtheriæ, to prove :

1. Cultivate on inspissated blood-serum twenty-four hours at 37° C. Note small white, dry-looking colonies. Prepare two cover-slides. Stain (1) with Löffler's methylene blue only, and (2) with Neisser or modified Neisser's stain. Notice on (1) irregular shape of bacilli, with well-marked granules, staining deeply with blue. On (2) granules appear black, whilst bodies of bacilli are stained a dull red colour.
2. Inoculate a tube glucose peptone broth. Incubate for twenty-four hours at 37° C. Observe formation of acid.
3. Prepare a cover-slide in ordinary way, dry, and drop, film side downwards, on to a glass slide on which has been placed a drop of dilute Löffler's methylene blue (1 to 5); press firmly down on to filter-paper, with cover-slide downwards; examine at once; apply 5 per cent. acetic acid by allowing to run under cover-glass. The organisms become partly decolorized, but the granules stand out distinctly as blue-black dots (Cobbett and Graham Smith's method).
4. Inoculate a guinea-pig with 1 c.c. of a forty-eight hours broth culture, and make a post-mortem. Note the local œdema at site of inoculation, and bacilli local only; look for a membrane on mucous surfaces.
5. The conclusive test is carried out as follows :

Inoculate two guinea-pigs—

- (1) With lethal dose of cultivation only.
- (2) With lethal dose ; also a protective dose of antitoxin.

The control animal No. 1 dies, whereas No. 2 is unaffected.

6. Stains, carbolic methylene blue, Gram, and as above.

Gram negative = *B. of Friedländer*, *B. influenzae*, *B. pestis*, *B. dysenteriae*, *B. mallei*, *B. lactis aerogenes*.

Gelatine at 20° C. : No growth, probably *B. influenzae*, *B. Ægypticus*, or *B. duplex lacunatus* ; growth and no liquefaction, *B. pestis*, *B. dysenteriae*, *B. Friedländer*, *B. mallei*, and *B. lactis aerogenes*.

B. influenzae, to prove :

1. Cultivate on blood-agar at 37° C. for twenty-four hours. Colonies look like drops of dew on surface.
2. Cover-slide : very minute rods ; do not grow in chains ; no capsules ; Gram negative ; no spores ; stain generally very badly, and often darker at either end.
3. No growth agar at 37° C. or gelatine 20° C., and generally very difficult to grow and cultivate ; cultures, if made even on blood-agar, soon die. Strictly aerobic, and powers of resistance generally very low.
4. Stain best with weak carbol-fuchsin.

B. Ægypticus (*Koch-Weeks Bacillus*).—Very minute rod ; non-motile ; Gram negative ; no spores ; no capsule. Closely resembles *B. influenzae*, but conditions

of growth even more restricted. Will rarely grow on blood-agar. Serum agar is the best media for cultivation; colonies very small, transparent, and resemble drops of dew.

Stain, weak carbol-fuchsin.

B. duplex lacunatus (*Morax-Axenfeldt Bacillus*).—Small, square-ended, plump, and usually diplobacillus; non-motile, no spores, and Gram negative. Will not grow on gelatine or agar media. Serum cultures show small rounded colonies which produce small cups of liquefaction; hence its name.

Stain, weak carbol-fuchsin.

Gelatine at 20° C. for forty-eight hours: Growth and no liquefaction, probably *B. pestis*, *B. dysenteriae*, *B. Friedländer*, *B. mallei*, *B. lactis aerogenes*.

Apply indol test. If positive = *B. Friedländer*, as other organisms in above group do not form indol.

B. pneumoniae (*B. of Friedländer*):

1. Cultivate on blood-agar at 37° C.
2. Peptone gelatine stab: incubate at 20° C.
Notice characteristic *white nail* appearance.
3. Prepare gelatine shake preparations. Incubate at 20° C. four days, and look for gas-formation.
4. Inoculate rabbit or mouse, and make smear preparations from heart-blood. Note that it is a diplobacillus, and is capsulated. Demonstrate the capsules by means of coloured ground substance, if original exudate is available.

5. Prove indol-formation.
6. Prove active fermenting powers on sugars.
7. Stain, carbolic methylene blue or weak carbolfuchsin.

Indol Test. Negative = *B. pestis*, *B. dysenteriae*, *B. mallei*, *B. lactis aerogenes*.

Litmus Milk. (1) Acid clot, and gas = *B. lactis aerogenes*.

(2) Alkaline, no clot, no gas = *B. dysenteriae*.

(3) Negative = *B. pestis* and *B. mallei*.

***B. lactis aerogenes*, to prove :**

1. Gelatine at 20° C. : abundant white growth, and gas-formation in shake culture.
2. Forms both acid and gas in lactose incubated at 37° C. for twenty-four hours.
3. Stain, carbolic methylene blue.

Resembles *B. coli*, but can be distinguished by being non-motile and absence of indol formation.

***B. dysenteriae*, to prove :**

1. Cultivate nutrose agar at 37° C. (no spores).

	Shiga-Kruse Type.	Flexner Type.
2. Glucose broth	+	+
Mannite „	0	+

Also see table of sugar reactions, p. 19.

3. Litmus milk. Note that the reaction is first acid, alkaline later, and no coagulation.
4. Microscopically this organism closely resembles *B. typhosus*, but is distinguished from it by—

- (1) Being non-motile (Shiga-Kruse) and sluggishly motile (Flexner), whereas *B. typhosus* is very actively motile.
 - (2) Ferments mannite (Flexner).
 - (3) Agglutination reaction is most important as a means of diagnosis.
5. Stain, carbolic methylene blue, or weak carbol-fuchsin, or Gram.

B. pestis, to prove :

1. Cover-slide : short bacillus, with rounded ends. Looks almost oval ; Gram negative ; shows polar staining ; no spores.
2. Haffkine's stalactite growths. Inoculate some peptone broth, to which some native butter or cocoanut oil has been added before sterilization ; incubate at 37° C. for three to six days ; observe long thread-like growths hanging down into clear media from under surface of butter.
3. Inoculate guinea-pig or rat, and recover bacillus from spleen and glands.
4. Stain, carbolic methylene blue, weak carbol-fuchsin, Bismarck brown.

B. mallei (glanders), to prove :

1. Potato : note honey-like growth, and media stained greenish-yellow when incubated at 37° C.
2. Cover-slide : small rods slightly curved, with rounded ends, and often showing well-marked plasmolysis ; no spores.

3. Strauss's reaction. Inoculate a male guinea-pig with a culture. Kill after forty-eight hours, and note acute inflammation of tunica vaginalis.
4. Mallein reaction ; test is usually applied to horses, and if carefully performed is diagnostic, but the essential thing is a rise of temperature of at least 1° , with the formation of an inflammatory area of definite size at the site of inoculation. (For details, see larger textbooks.)

Stain, carbolic methylene blue, methyl violet, or weak carbol-fuchsin.

NON-PATHOGENIC ORGANISMS RESEMBLING PATHOGENIC ONES

Micrococcus lineæ, usually supposed to be concerned in the reduction of urea to ammonium carbonate, is by many observers held to be identical with *Staphylococcus albus*.

Micrococcus candicans resembles *Staphylococcus albus*, but is distinguished by *not* liquefying gelatine.

Sarcina alba resembles *Staphylococcus albus* in growth, but microscopically is larger, and always in tetrads.

Sarcina aurea and *S. lutea* resemble *Staphylococcus aureus* and *S. citreus* microscopically, but are distinguished by being larger in size and arranged in tetrads.

Micrococcus agilis resembles staphylococci, but growth is deep red, and organism is motile.

Streptococcus faecalis, *S. equinus*, *S. brevis*, resemble *S. longus*, but chains are shorter, and broth culture is a uniform turbidity instead of sandy deposit and clear broth.

Bacilli (Gram positive).

Bacillus Hoffmanni resembles *B. diphtheriæ*, but is distinguished by the shorter length, staining evenly, and usually occurring in pairs joined at their bases. They resemble one large torpedo-shaped bacillus

with a clear zone in the middle. They are usually Neisser negative, and show palisade arrangement. Cultures = no acid in dextrose broth.

B. xerosis sometimes closely resembles *B. diphtheriae* microscopically, but is negative in dextrose broth, and stains evenly.

B. mycoides closely resembles anthrax, both culturally and microscopically, but is motile. Inoculation is the only deciding point in absolute diagnosis.

B. subtilis resembles above, but is actively motile, and ends are often rounded.

Organisms resembling *B. Tuberculosis* in their Acid-fast Characters.

B. smegmatis, distinguished by being decolorized by alcohol after acid.

Butter bacillus, distinguished by rapid growth on artificial media.

B. phlei, distinguished by rapid yellow growth at 20° C.

Manure bacillus (mist bacillus), distinguished by rapid yellow growth at 20° C.

B. pseudo-tuberculosis, distinguished by Gram negative and non-acid fast.

Several varieties of cladothrix resemble actinomycosis, but all grow much more readily and branch more freely. Show false branching. Gram negative.

B. fluorescens liquefaciens resembles microscopically *B. pyocyaneus*, but grows more readily at 20° C. than at 37° C. *B. fluorescens non-liquefaciens* resembles above, but does not liquefy gelatine. Both give only a green fluorescence to media, and not the gradually darkening green colour of *B. pyocyaneus*.

BRIEF SUMMARY OF PRINCIPAL STAINING METHODS

Simple Stains.

Carbolic Methylene Blue (C.M.B.).

1. Prepare a cover-glass film by spreading a thin layer of diluted culture over cover-glass with platinum loop ; dry in fingers (to avoid over-heating) over Bunsen flame.
2. Stain C.M.B. four minutes.
3. Wash well in water.
4. Dry between folds of filter-paper first, afterwards by passing once or twice through Bunsen flame in fingers.
5. Mount in xylol Canada balsam.

Simple Stains in Common Use.

Violet—Methyl violet, gentian violet, crystal violet.
Blue—Methylene blue, Victoria blue, thionin blue.
Red—Carbolic fuchsin, eosin, safranin, neutral red.
Brown—Bismarck brown.
Black—Silver stains oxide or pyrogallate methods.
Green—Dahlia and malachite green.

Compound Stains.

Gram's Method.

1. Prepare film in usual way.
2. Stain aniline gentian violet three minutes.
3. Wash in water.
4. Treat film with Gram's iodine solution until black.
5. Wash in water.
6. Decolorize with alcohol (until no more stain washes out).
7. Wash in water.
8. Counter-stain neutral red one to five minutes.
9. Wash in water.
10. Dry and mount.

Ziehl-Neelsen's Method (for acid-fast organisms).

1. Prepare film in usual way.
2. Stain hot carbol-fuchsin for five to ten minutes.
3. Wash in water.
4. Treat in 25 per cent. sulphuric acid (about five seconds).
5. Wash in water.
6. Flush slide with absolute alcohol.
7. Wash in water.
8. Counter-stain C.M.B. two minutes.
9. Wash in water.
10. Dry and mount.

Modified Neisser Stain (Clinical Research Association).

1. Prepare film in usual way.
2. Apply Neisser I. stain for two minutes.
3. Wash in water.
4. Apply Gram's iodine solution for ten seconds.
5. Wash in water.
6. Apply 0.25 per cent. neutral-red stain for one minute.
7. Wash in water.
8. Dry and mount.

NOTE.—In the writer's opinion the above stain gives better results than the original Neisser method, as the neutral red gives a better background than the Bismarck brown in Neisser II.

Richard Muir's Method.

1. Prepare film in ordinary way, and dry well.
2. Stain carbol-fuchsin for about thirty seconds, heating gently.
3. Wash slightly with spirit.
4. Wash well with water.
5. Place in mordant for few seconds.
6. Wash well in water.
7. Immerse in methylated spirit for one minute.
8. Wash well in water.
9. Counter-stain watery solution methylene blue for half a minute.
10. Wash well in water.
11. Dehydrate with alcohol.

12. Clear with xylol and mount in xylol balsam.
The bacteria are a deep crimson, and the capsules of a blue tint.

Staining for Spores.

1. In using ordinary watery solutions of basic aniline dyes, such as methylene blue, the bacilli take up the stain readily, but the spores remain as clear unstained spheres, an old culture of anthrax bacilli, for instance, showing this remarkably well.

2. *Muir's Method* (modified).

1. Stain cover-glass films as for tubercle bacilli.
2. Decolorize with alcohol 2 parts, acetic acid (1 per cent.) 1 part, or methylated spirit. (This removes the stain from the bacilli.)
3. Wash in water. (Place under $\frac{1}{8}$ -inch objective mounted in water ; observe if spores are red and remainder of films colourless. If satisfactory, proceed as under.)
4. Stain watery solution methylene blue (half a minute).
5. Wash in water.
6. Dry and mount in xylol balsam.

Spores are stained red, bacilli are stained blue.

Staining for Flagella.

1. Prepare film by mixing a very small quantity of a young culture (eighteen-hours broth at 37° C.) with water on cover-slide ; dilute a second time by taking a very small quantity from first cover-slide and mixing

with a drop of water on second slide. Spread out second slide with a needle, allow to dry in air; finish off by passing through Bunsen flame twice. Be careful not to overheat.

2. Pour mordant solution on to prepared film. Heat gently until steam rises for one minute.
 3. Wash well in water two minutes.
 4. Dry film carefully over flame.
 5. Pour stain on to cover-slide. Heat as for mordant one minute.
 6. Wash well in water.
 7. Dry and mount.
- (Pitfield's method as modified by R. Muir.)

Stains for Blood-Films.

Leishman's Stain.

1. Prepare thin blood-film (unfixed).
2. Apply Leishman's stain for one minute.
3. Dilute by applying double the quantity of distilled water to stain on slide, and mix rapidly. Allow to stand diluted ten minutes.
4. Wash well in distilled water.
5. Dry and mount.

Giemsa's Stain for Spirochæta.

1. Prepare film as described under Spirochætes.
2. Fix methylic alcohol five minutes.
3. Apply diluted stain fifteen to twenty minutes.
(Use 1 drop of stain in 1 c.c. distilled water.)

4. Wash in distilled water.
5. Dry and mount.

Note that the above stain answers admirably for smear preparations, but for sections of tissue, organs, etc., the silver pyrogallate method is recommended. For details of method see Muir and Ritchie's "Bacteriology," but the following is brief summary of process :

Staining of Spirochæta in Sections of Tissues.

1. Cut sections in slices 1 millimetre in thickness.
2. Place in 10 per cent. formalin solution for twenty-four hours.
3. Wash for one hour in water.
4. Place in 96 per cent. alcohol for twenty-four hours.
5. Place in a 1.5 per cent. solution of silver nitrate in dark green or amber bottle, and place in incubator at 37° C. for three days.
6. Wash in water for twenty minutes.
7. Place in a 4 per cent. pyrogallic acid solution in distilled water, to which has been added 5 per cent. formalin; keep in a dark bottle for forty-eight hours.
8. Wash well in water.
9. Take through alcohol in increasing strengths up to absolute, keeping in each strength of alcohol for twenty-four hours.
10. Sections are then embedded in paraffin, and mounted as described under Examinations of Organisms in Tissues.

(Ramon y Cajal and Levaditi's method modified.)

The spirochætes, if present, are a black colour, and

show up against the pale yellow background of the tissues. Weak carbol-fuchsin or neutral red can also be used to stain the background, if desired.

Examination of Organisms in Tissues.

1. Cut tissue into small cubes not more than $\frac{1}{8}$ inch thick.

2. Harden in methylated spirit for twelve hours.

3. Transfer to absolute alcohol for twenty-four hours.

4. Then to mixture of absolute alcohol and chloroform twenty-four hours.

5. Then pure chloroform twenty-four hours.

6. Transfer to mixture chloroform and paraffin, and keep melted in or over oven twenty-four hours.

7. Finally pure melted paraffin twenty-four hours.

8. Cast tissue in a block with paraffin.

9. Cut sections with microtome, floating off after cutting into ice-water.

10. Float section on to glass slide moistened with few drops dilute alcohol; place in warm incubator three or four hours.

11. Remove paraffin with xylol or chloroform.

12. Remove xylol with alcohol.

13. Remove alcohol with water.

14. Apply stain in usual way—Gram, Ziehl-Neelsen, Giemsa, etc.

15. Remove water after staining with alcohol (or with aniline oil method).

16. Remove alcohol with xylol:

17. Mount in xylol balsam for permanent section.

To Stain Permanently a Hanging-drop Specimen.

Occasionally it is necessary to obtain a permanent record of a hanging-drop specimen, particularly if there is any special grouping of the organism, such as the long chain of the cocci in *Streptococcus longus* or *conglomeratus*; these could not be seen in a colony from the agar growth, as removal with the loop usually spoils the special grouping.

The method is as follows :

1. Remove the cover-slip from alcohol (in which slips should always be stored), and pass through Bunsen flame, allowing the alcohol adhering to it to burn off.
2. Place a large loop of the emulsion or broth culture on the cover-slide.
3. Spread evenly over cover-slide with a needle previously sterilized.
4. Place on rubber mat and allow to dry in the air, covering over the specimen with a watch-glass to exclude dust, etc.
5. Pass film twice through Bunsen flame held in the fingers.
6. Immerse film in alcohol (or corrosive sublimate solution) five to ten minutes, and allow to dry.
7. Saturate with acetic acid 2 per cent. for two minutes.
8. Wash well with alcohol, and allow some to remain on cover-slide for two minutes.
9. Wash well with water.
10. Stain as for ordinary films.

Nos. 7 and 8 are necessary in order to clear the background and give a much sharper and clearer film, especially if gelatinous substances are present.

Preparation of Blood-Films for Staining.

Modified Christopher's and Stephens' Method.

1. Prick the finger or lobe of the ear after cleaning with alcohol, and express a droplet of blood.
2. Take this up by touching with a clean glass-slide about an inch from one end.
3. Lay a sterile needle across the slide and on to the blood.
4. Wait until the blood has run out by capillarity between the needle and the slip.
5. Then, holding the needle by one end, push it over the surface of the slip, in the direction of the length of the latter, in order to spread the blood over the entire breadth of the middle of the slip.
6. Allow film to dry in air.
7. Fix with alcohol, and dry.
8. Stain with differential stain.

Jenner's, Leishman's, and Giemsa's are all very suitable.

The body of the malarial or other parasite is stained blue, and the chromatin of the nuclei ruby-red.

NOTE.—It is not necessary to cover these slides with a cover-slip unless they are to be preserved, as a drop of cedar oil direct on the film is sufficient for examination by means of a $\frac{1}{12}$ -inch objective.

Thick Film Method for Rapid Detection of Malarial and Other Blood-Parasites (Ross).

1. A large drop of blood is placed upon a glass slide, spread out slightly to form a thick film.
2. Allow to dry in air.
3. Place film carefully in aqueous eosin solution fifteen minutes.
4. Wash very gently in water.
5. Place in weak solution of methylene blue for a few seconds only.
6. Wash very gently with water.
7. Dry and mount in Canada balsam.

This preparation has the advantage over the ordinary method inasmuch as 20 cubic millimetres of blood can be examined at one time. Malarial parasites appear as blue rings (protoplasm) with a deep crimson blot within or upon the rings (chromatin).

The above method can be further simplified by simply staining thick blood-films with Giemsa or Leishman stain after removing hæmoglobin as for filaria.

To Examine Fresh Blood for Amœboid Movements of White Corpuscles, or to Examine any of the Various Varieties of Amœbæ for Pseudopodia, Amœboid Movements, etc.

For blood :

1. Place a drop of blood and a drop of 0·7 per cent. saline solution in a cover-slide.
2. Invert on to a glass-slide, and do not press down.

3. Place on stage of microscope arranged with a projecting metal arm.

4. Apply small flame of Bunsen burner to tip of projecting arm.

5. Regulate temperature of stage by placing a thermometer on the stage ; keep the temperature as near as possible about 37° C.

6. Observe with $\frac{1}{8}$ -inch objective and rack-condenser down so that the light is not too bright.

The above method is not suitable when a prolonged observation is required, as the moisture quickly evaporates ; but if an observation is required extending over a considerable period, the ordinary hanging-drop process answers well if a larger drop than usual is used, and spread over a larger area with a needle before applying to the vaseline ring.

A special warm stage through the interior of which water at any temperature required can be circulated is extremely useful for this class of work, as it can be fixed on to the microscope stage when required.

For amœbæ and small parasites in fæces or other material, it is best to shake up the material with weak saline solution or ordinary tap-water in a test-tube ; allow to stand for some time, and remove the upper layers of fluid and débris ; add more water, and centrifugalize the contents of tube.

Finally, remove the upper layers of water with a pipette, and examine the deposit as described above.

SPECIAL REACTIONS

A VERY brief outline is given here of several of the more important bacteriological processes at present in use in the practice of medicine for diagnostic or other purposes. They are placed here for reference only, to enable the student to quickly refer to the technique whilst actually doing the processes, and are in no way to be considered as completely descriptive.

Wassermann's Reaction.

This reaction is now so commonly employed in diagnosis that it is essential that students should be familiar with a general outline of its technique.

The test depends on a reaction known as "complement fixation." For an explanation of this term a textbook should be consulted.

The essentials for carrying out the test are as follows :

1. **Antigen.**—This consists of an alcoholic extract from the liver of a syphilitic fœtus. The liver is extracted with four times its weight of alcohol. After filtration through filter-paper it is placed in an ice-chest. A precipitate forms which sediments; this sediment should not be disturbed when taking antigen for use.

The strength of the antigen becomes constant after about ten days.

NOTE.—There are various methods of preparing antigens with saline or other solvents, also by using guinea-pig's heart muscle instead of syphilitic liver ; but one process only is given, for the sake of simplicity and brevity.

2. **Patient's Serum.**—It is most convenient to draw off 5 c.c. of blood from the median cephalic or median basilic vein, using a sterile all-glass syringe with aseptic precautions. The blood is squirted into a sterile centrifuge tube, and placed in the ice-chest for half an hour. The clot is then broken up, the tube centrifugalized, and the clear serum pipetted off, and placed in a water-bath at 56° C. for thirty minutes to destroy the complement.

3. **Complement.**—This is most easily obtained from a guinea-pig. As the complement is a very unstable substance, the pig must be killed as shortly as possible before carrying out the test. The blood is best obtained by inserting a sterile glass pipette into the right ventricle and sucking up the blood. With practice about 3 c.c. can be obtained. The blood is then transferred to a sterile centrifuge tube, and placed in the ice-chest for half an hour ; finally the clot is broken up and centrifugalized. The serum thus obtained is pipetted off into a sterile test-tube.

4. **Erythrocyte Solution.**—For this solution washed sheep's corpuscles are generally used, but as in many cases this is inconvenient, human blood corpuscles can be used equally well, the essential thing being to use the *same* corpuscles as have been used to

produce the hæmolytic serum in the rabbit (see below).

The fresh blood is received into 0·75 per cent. saline solution defibrinated by shaking well, and the corpuscles washed by centrifugalizing with successive quantities of fresh saline solution. (*Note*.—If only a small quantity of blood is available, it is unnecessary to wash the corpuscles, but allow about ten drops of blood to fall direct into about 20 c.c. of the saline solution.)

5. Hæmolytic Serum.—This is obtained by inoculating a rabbit with either human or sheep's corpuscles washed in saline solution. It is best to start with an inoculation of 2 c.c., a second of 3 c.c., and subsequent inoculations of 5 c.c. of blood-corpuscles. A small quantity of sterile 1 per cent. solution of sodium citrate placed in the syringe will prevent clotting when obtaining the blood in the first instance.

The author usually gives six inoculations into a fair-sized rabbit at intervals of eight or ten days. The serum is obtained from the vein which runs along the posterior border of the rabbit's ear. The blood is caught in a centrifuge tube, placed in the ice-chest for half an hour and allowed to clot; the clot is then broken up, the tube centrifugalized, and the clear serum pipetted off. The serum is finally placed in a water-bath at 56° C. for half an hour, in order to destroy its natural complement.

The hæmolytic serum is then standardized. It is not possible to give a detailed description of the process for standardizing the various solutions used for the tests; practically it consists of a series of preliminary

tests with various dilutions of the reagents until a satisfactory complete hæmolysis is obtained.

We have now the following solutions ready for carrying out the reaction :

1. Antigen -alcoholic solution of syphilitic-liver infecting material.

2. Patient's serum to be tested.

3. Complement in guinea-pig's fresh serum.

4. Erythrocyte solution (washed blood-corpuscles).

5. Hæmolytic serum (from immunized rabbit).

6. Sterile saline solution.

7. If possible, some serum from a known syphilitic patient as control.

There are several ways of carrying out the tests as regards the contents of the various test-tubes, but the following is a brief description of a method that has given good results in the author's hands :

Prepare six sterile test-tubes marked A, B, C, D, E, and F.

A. Tube prepared as follows :

1. Antigen, 0.2 c.c.

2. Patient's serum, 0.2 c.c.

3. Guinea-pig complement, 0.1 c.c.

4. Saline solution, 2 c.c.

Place in incubator for one hour, and add—

5. Hæmolytic serum (diluted to strength) 0.2 c.c.

6. Washed blood-corpuscles in saline, 1 c.c.

Place in incubator for two hours at 37° C. (eight hours in cold).

B. As above, using a known syphilitic serum (for 2).

C. As above, using a normal non-syphilitic serum (for 2).

D. As above, but omit antigen.

E. As above, but omit hæmolytic serum.

F. As above, but omit patient's serum.

RESULT.

If Syphilitic.

A. No hæmolysis.

B. No hæmolysis.

C. Hæmolysis.

D. Hæmolysis.

E. No hæmolysis.

F. Hæmolysis.

If not Syphilitic.

A. Hæmolysis.

B. No hæmolysis.

C. Hæmolysis.

D. Hæmolysis.

E. No hæmolysis.

F. Hæmolysis.

Widal's Reaction.

Microscopical Method.—1. Prepare a dilution of original serum (1 part to 9 parts 0·7 per cent. sterile saline solution), using a capillary pipette. Label A = 1 in 10 dilution.

2. Prepare a dilution of 1 part A to 9 parts saline as before. Label B = 1 in 100 dilution.

3. Place on a cover-slide one loopful of the diluted serum A, one loopful of a twenty-four hours' culture of B.T.A. (which has been growing twenty-four hours at 37° C. in bouillon). Result = dilution of 1 in 20 (5 per cent.).

4. Repeat process with B. One loopful of B and one loopful of *B. typhosus abdominalis* culture = dilution 1 in 200 ($\frac{1}{2}$ per cent.).

5. Prepare a hanging-drop preparation of the B.T.A. broth culture *without* any serum as control—C.

6. Make hanging drops of A, B, and C, and place

each under separate microscope, using $\frac{1}{6}$ -inch objective and racking condenser well down; also partly close diaphragm.

7. Compare them at end of fifteen minutes, and again at end of thirty minutes, looking for clumping and decrease in motility.

Macroscopical Method.—1. Prepare dilutions as above.

2. Mix with B.T.A. culture by drawing up into pipette with an equal amount of diluted serum.

3. Seal off capillary end, and allow to stand twenty-four hours.

4. Examine by naked eye and hand-lens for flocculent masses of agglutinated bacilli.

The above agglutination methods can be applied to almost any organism, and are not peculiar to *B. typhosus*, although this is best known. It is a most valuable method of differentiation between closely allied organisms, especially in high serum dilutions.

To Take an Opsonic Index.

Apparatus Required.—1. Prepare some washed blood-cells by centrifugalizing citrated blood repeatedly with sterile saline solution, 0.7 per cent.

2. Sample of patient's blood (centrifugalized).

3. Sample of normal blood (centrifugalized) as a control.

4. Emulsion of organism well shaken and free from clumps.

5. Capillary pipette marked about $\frac{1}{4}$ inch from end, and fitted with teat.

Method.—For example, process is given for tubercle.

1. Take up 3 parts in capillary pipette of washed white blood-cells, 2 of T.B. emulsion, 2 of patient's serum.

2. Mix thoroughly by expelling and drawing up into pipette several times.

3. Draw away from capillary end, and seal in flame.

4. Mark pipette with number or patient's name.

5. Prepare an identical preparation, using the normal serum.

6. Incubate both for twenty-five minutes at 37° C.

7. Prepare blood-films from both by breaking capillary end off and blowing out on to slide, spreading with another slide.

8. Stain Ziehl-Neelsen's method, and counter-stain thionin blue.

9. Count the number of T.B. present in fifty polymorphonuclear cells on each slide, and work out index.

EXAMPLE.—A slide contained 20 T.B. in fifty polymorphonuclear cells.

Normal control slide contained 30 T.B. in fifty polymorphonuclear cells.

$$\therefore \text{index of A. } \frac{20}{30} \text{ or } 0.66.$$

The same process applies to taking the opsonic index to *any* organism, the only difference being that in most other organisms the mixture is—cells 2, emulsion 1, serum 1, and Leishman's stain is used to stain the films, after incubating for fifteen minutes at 37° C.

Tuberculin Reaction consists in injecting a small (0.001, 0.005, and 0.01) dose of Koch's old tuberculin (T.O.A.) into a suspected patient. The reaction is positive if the temperature rises from 1° to 1.5° or higher within twelve hours, the patient during this time remaining under unaltered conditions.

Calmette's Reaction consists in applying a drop of a very weak solution of glycerine-free old tuberculin (Koch) to the conjunctiva, either 0.5 or 1 per cent. It is positive if an acute conjunctivitis follows within thirty-six hours, which is painless and soon passes off.

Von Pirquet's Reaction consists in scarifying the surface of the skin, as in vaccination, and applying old tuberculin solution (10 to 20 per cent.). If positive, a localized dusky red papule supervenes.

Pure Cultures.

To prepare a pure culture from a mixed culture containing several organisms, there are many processes used by bacteriologists, mostly varied so as to separate special groups of organisms—for example, the separation of the coli-typhoid groups by means of Conradi-Drigalski medium or neutral-red mannite plates, etc. But a simple separation, which can be applied by any student, is as follows :

First prepare three nutrose-agar Petri dishes, and dry for two hours at 42° C. Add a few c.c. of 0.7 per cent. sterile saline solution to the culture in the tube—for example, a mixed growth of *Bacillus coli* and

streptococci on nutrose agar which has been incubated twenty-four hours at 37° C. ; loosen colonies with platinum loop (sterilized in flame); shake up the saline well, so that the growth on surface of medium is well emulsified ; take a loopful of the emulsion and place on surface of plate marked 1. Now with a sterile grass spreader, L-shaped (or bend the handle of platinum loop, and sterilize in Bunsen flame), spread the loopful of culture all over the surface of agar plate ; then with the same spreader over plate 2 and plate 3 in succession. Do not add any more of the original emulsion, the amount clinging to the spreader being sufficient for 2 and 3. Incubate the plates 1, 2, and 3 at 37° C. for twenty-four hours, placing the plates with agar surfaces above in incubator (*i.e.*, inverted). The different colonies can now be picked out on plates 2 and 3, and planted on nutrose-agar sloped tubes. These are now incubated and examined for pure cultures microscopically, etc. The reason three plates are used is because the colonies on plate 1 are usually too thick to be picked off easily with platinum loop separately ; they are less thick on 2, and usually discrete and well defined on 3.

Standardization of Bacterial Emulsions.

It is often necessary to count the number of bacteria present in a c.c. of an emulsion, usually with a view to preparation of a vaccine of known strength. This can be done by either of the following methods :

1. By making various dilutions of the emulsion, and then adding $\frac{1}{10}$ c.c. to gelatine or agar plates ;

incubate and count number of colonies present after twenty-four hours; calculate from number of bacilli present in $\frac{1}{10}$ c.c. of diluted emulsion the number of bacilli present in original emulsion in $\frac{1}{10}$ and then in 1 c.c. The counting of the colonies present on the plate is most conveniently done by using a Pakes disc. This method is more accurate than the following, but is not suitable for vaccines where the numbers are wanted quickly and absolute accuracy is not so important.

2. Prepare a capillary pipette marked about $\frac{1}{2}$ inch from its extremity, some 1·5 per cent. sterile sodium citrate solution, the bacterial emulsion as given under Pure Cultures, two glass slides.

Method.—1. Draw up 2 parts citrate solution into pipette.

2. Prick the finger, and draw up 1 part fresh blood.

3. Draw up 1 part emulsion into pipette.

4. Mix well together by expelling on to slide and drawing up into pipette several times.

5. Expel on to slide, and spread evenly over the surface with other slide.

6. Allow to dry in air; finish over flame.

7. Stain with Leishman's stain.

8. Count with Ehrlich's counter or square marked on eyepiece.

9. Count the number of red blood-cells and the number of bacteria in each field for ten fields; then total up cells and organisms.

10. Work out number of organisms per c.c. as follows:

Method of Counting Organisms.

The method consists in counting the number of organisms against the number of red corpuscles in normal blood, which are known and fairly constant, a variation of a hundred or so, or even more, making very little difference when working with such large numbers.

For example, normal blood contains 5 millions red corpuscles in 1 cubic millimetre, or 5,000 millions per c.c. In the example to be counted the total of ten fields was, say, corpuscles 300, and organisms 200.

Therefore $\frac{200 \times 5,000 \text{ millions}}{300} = 3,333 \text{ millions}$

organisms per c.c. of the emulsion. This emulsion of known strength can now be diluted with sterile water to any number of organisms per c.c. required as follows:

For example, the emulsion is found to contain 3,300 millions of organisms per c.c. Assuming that the doses of organisms required for treatment are those of 1,000, 500, 250, 100, 50, 10, and 5 millions in *each* dose, the dilution could be made as follows :

One c.c. of original emulsion added to 2.3 c.c. of sterile water gives an emulsion containing 1,000 millions per c.c. Label this tube A, and make further dilutions, using sterile water and sterile pipette, as follows :

1 c.c. of A with 1 c.c. sterile water = B, containing 500 millions per c.c.

1 c.c. of B with 4 c.c. sterile water = C, containing 100 millions per c.c.

1 c.c. of C with 9 c.c. sterile water = D, containing 10 millions per c.c.

The emulsions can now be put up into sterile vaccine vials, using a sterile syringe and needle, the piston-rod of which is graduated with $\frac{1}{2}$ c.c. graduations. For example :

1 c.c. of A contains 1,000 millions of the organism.

$\frac{1}{2}$	"	A	"	500	"	"	"
$\frac{1}{2}$	"	B	"	250	"	"	"
1	"	C	"	100	"	"	"
$\frac{1}{2}$	"	C	"	50	"	"	"
1	"	D	"	10	"	"	"
$\frac{1}{2}$	"	D	"	5	"	"	"

The above doses are usually sufficient for any ordinary organism, but by appropriate dilution *any* number of organisms can be put up as a vaccine.

As soon as the doses are measured into the vials, the necks are sealed off in a Bunsen flame. A different-coloured glass may be used for each strength of vaccine, and the number of organisms present in the dose marked on the vial also.

The original emulsion is killed by placing in water kept at 60° C. for one hour, after sufficient has been drawn up into the capillary pipette for the purpose of counting ; and when the vials containing the various doses have been sealed off, they are again sterilized by heating to 60° C. for one hour.

In practice it is preferable to put up the doses in $\frac{1}{2}$ c.c. of fluid rather than 1 c.c., as the injection of the fluid subcutaneously causes more pain to the patient than the actual puncture with the needle ; therefore, the smaller the actual quantity of fluid to be injected, the less inconvenience is caused.

NOTE.—After diluting the original emulsion with sterile water, and before putting up into vials, a drop of trichlor-cresol is added to each tube, with the view of inhibiting the growth of any air-borne organisms that may contaminate the emulsion during manipulation.

To Make a Blood Culture from a Living Human Subject.

This method is employed with a view to determine the presence of organisms in the blood.

Apparatus Required.—Sterile all-glass syringe of 10 c.c. capacity.

Sterile needle to fit on nozzle of same.

Two c.c. of sterile citrate solution in a capsule.

Indiarubber tourniquet.

Six tubes of sterile nutrient broth.

The method is as follows :

1. Thoroughly clean up patient's arm over the anterior surface of forearm with lysol and warm water.

2. Clean well with warm water and ether soap.

3. Wash thoroughly again with pure ether.

4. Apply tourniquet to upper arm well above the elbow.

5. Adjust the same so as to cause venous congestion, but not to stop the arterial blood flow.

6. Fix the needle to nozzle of the syringe, and draw up into syringe the citrate solution from the capsule, being careful to expel all air.

7. Insert the needle of the syringe into the distended median basilic or median cephalic vein.

8. Allow the syringe to fill slowly with blood, easing the piston gently to assist the flow.

9. When sufficient blood has been collected, remove the tourniquet and withdraw the needle.

10. Place a large square of Mead's strapping over the site of puncture, and keep in place with a bandage.

11. Inoculate each of the six tubes of broth with equal quantities of the blood at once.

12. Place the cultures in the incubator, and incubate at 37° C.

13. Examine the cultures at twenty-four, forty-eight, and seventy-two hours in the usual manner for the presence of organisms.

STERILIZATION

As this subject is of such extreme importance in bacteriology, a brief outline of the principal methods in use is not out of place even in such a small volume as the present. As applied in the Bacteriological Laboratory, many of them, as sunlight, electricity, etc., are useless; whilst others, such as chemical reagents, are limited in their application, and practically the latter are only used to fill the troughs, jars, etc., into which infected slides, pipettes, tubes, etc., are thrown before being washed preparatory to being again sterilized.

The principal methods in use are :

Dry Heat.—1. Naked Bunsen flame, as used for platinum loops, needles, scissors, and forceps during the process of a post-mortem.

2. Muffle furnace, used chiefly for sterilizing porcelain, filter-candles, and various filtering media.

3. Hot-air oven, as used for glass and metal substances generally. Hot air at 150° C. destroys all bacteria, spores, etc., in about thirty minutes, whilst a few minutes' exposure to 180° C. will effect the same result.

But as its penetrating power is not great, hot air should not be used for large masses of fabric.

Moist Heat.—1. Water at 60° C. This method is usually employed for albuminous fluids, such as the killed bacterial emulsions used in preparing vaccines. It is most effective if the substance to be sterilized is placed in the water-bath, kept at 56° C. to 60° C. for one hour, then taken out and allowed to cool. Any manipulations, such as diluting to specific strengths and putting up into doses, can now be carried out. Finally, the finished product is again returned to the water-bath for one hour.

Many workers prefer to wait until the bulbs have been hermetically sealed, others to sterilize twice.

The two heatings are necessary, as at 60° C. only the vegetative forms of bacteria are destroyed, the spores, if present, escaping; these would, however, assume the vegetative form when the conditions again become favourable—as on cooling, for example—and, being in the vegetative form, when heated for the second time, would be destroyed.

2. Water at 100° C. is chiefly used for the sterilization of surgical instruments, rubber tubing, syringes, etc.

3. Steam at 100° C., usually applied by means of Koch's sterilizer, or some modification of the same. Steam at 100° C. will destroy the vegetative forms of bacteria in fifteen to twenty minutes, but sporing forms require from one to two hours.

The most efficient way of applying the method is that known as the "intermittent," and consists of sterilizing by exposure to steam at 100° C. for twenty minutes on each of three consecutive days. This method is used principally in preparing sterile media.

4. Superheated and supersaturated steam at 115°C . to 120°C . This method is applied by the use of an autoclave, the steam being under pressure, and is the most effective way of dealing with infected textile fabrics, as it will destroy both the vegetative and sporing forms of bacteria in fifteen minutes.

It is customary, when using the larger forms of sterilizers for applying superheated steam, such as the Washington-Lyon, Thresh's, etc., to place a test-packet of infected material in the centre of the articles to be disinfected, such as dressings, infected clothes, etc. On removal from the sterilizer, small pieces of the lint, wool, or other material contained in the test-packet, are incubated in sterile nutrient broth for forty-eight hours at 37°C ., and a hanging drop examined for the presence of living organisms before the articles sent for sterilization are certified as sterile.

In practice, owing to the time required, this method is only applied to surgical dressings. But various indicators of a chemical nature, and also eggs, are frequently used as tests.

Filters.—1. Cotton-wool filters form a most efficient means of sterilizing air or gases, and are also used to plug sterile tubes used in the bacteriological laboratory, as, although air can pass through cotton-wool freely, the contained organisms are entangled in the meshes and retained. If kept *dry*, a cotton-wool plug remains efficient for a considerable time, but at once becomes useless if allowed to become wet.

2. Porcelain filters are used for the sterilization of liquids by filtration. They are of two kinds: the

porous porcelain, known as the "Pasteur-Chamberland," or those prepared of a fine diatomaceous earth, and known as the "Berkefeld." As the passage of liquids through both of these is extremely slow, it is usual to aid the flow by creating a partial vacuum in the receiving vessel, aspiration being thus employed to hasten the process.

Filters of this description are only effective for a very limited period, and must be sterilized in the muffle furnace before being again used, as bacteria rapidly grow through the pores.

They are very useful for the separation of soluble toxins.

Aseptic Method of Making a Cultivation, with a View of Isolating and Growing the Infecting Organism present in a Morbid Tissue sent for Bacteriological Examination.

Example.—A piece of lung or the spleen is sent for examination.

Apparatus Required.—A searing-iron heated to dull redness over a gas-stove.

Sterile scalpel and pair of forceps freshly boiled.

Sterile platinum loop, which is heated to redness in the Bunsen flame before use.

Four sterile culture-tubes, containing—

1. Nutrient broth.
2. Nutrient agar sloped.
3. Blood-agar sloped.
4. Blood-serum sloped.

Method.—1. Fix the tissue firmly to a piece of board.

2. Sear about 2 square inches of its surface well with the searing-iron.
3. Make one deep incision into the seared area with a scalpel.
4. Insert the points of sterile dissecting-forceps into the cut.
5. Sterilize the platinum loop, and, whilst holding open the sides of the incision with the forceps, take up as much of the substance at the bottom of the incision as possible on the loop.
6. Inoculate the broth tube with the loop.
7. Inoculate the agar, blood agar and serum tubes in the same manner, smearing the loop well over the surfaces of the media; flame the loop after each inoculation.
8. Flame the necks of the tubes before replacing plugs of wool.
9. Finally flame the plugs of wool.
10. Incubate at 37° C. for twenty-four hours, and examine for growth; replace in incubator, and again examine at forty-eight hours, and again after seventy-two hours.

NOTE.—If the piece of tissue sent for examination is too small to carry out the above process, it is best to cut it into small pieces with the sterile scissors, and then smear the cut surface of the actual tissue over the surface of the media by the aid of the sterile forceps, dropping one piece also into the broth tube.

Incubate and examine as above.

Post-mortem Examination of Experimental Animal.

NOTE.—The post-mortem should be carried out as soon as possible after death, or the tissues are rapidly invaded by bacteria, principally derived from the alimentary canal, usually the *Bacillus coli communis*.

The following utensils must first be sterilized before commencing the post-mortem, placed in readiness, and protected from contamination :

Three pairs of scissors.

Three pairs of forceps.

Three scalpels.

Spear-headed platinum spatula with hole in it.

Three searing-irons.

Platinum loop.

Sterile capillary pipettes and capsules.

The following articles should also be placed in readiness for use :

Tubes of media, bouillon, sloped agar, blood-agar, and blood-serum, together with cover-slips and glass-slides.

Do not expose the utensils in sterilizer until quite ready to begin the post-mortem, and keep the searing-irons constantly at a dull red heat in a Bunsen flame during the operation.

Method of Procedure.—1. Fasten the animal, ventral surface upwards, on a board with copper nails through the extremities.

2. Drench the animal and board with lysol 2 per cent.

3. With sterile forceps and scalpel make an incision through the skin from the top of the sternum to the pubes. Make lateral incisions from this to axillæ and groin, reflecting skin in two lateral flaps.

4. Inspect site of inoculation. If any local lesion is present, make cover-slide preparations, and inoculate culture-tubes from it.

5. Sear exposed surface of thorax with searing-irons.

6. Remove a rectangular portion, comprising practically the whole of the anterior chest-wall, to expose heart and lungs, using a pair of sterile scissors and forceps.

7. Burn through raised pericardium with searing-iron.

8. Grasp the apex of the heart with fresh pair of sterile forceps; sear the surface of right ventricle.

9. Plunge the open point of a capillary pipette through the seared surface into the right ventricle, and fill pipette with blood.

10. Make cultivations and cover-slip preparations of blood.

11. Sear a wide line down anterior abdominal wall, and open peritoneal cavity by an incision with fresh sterile scalpel down the centre of seared line.

12. Collect specimens of peritoneal fluid in sterile capillary pipette; make cultivations and cover-slip preparations of same.

13. With fresh pair of sterile scissors and forceps excise the spleen, liver, and kidneys, lungs, lymphatic glands, etc., or any organ that shows signs of infection or disease.

14. Place each in sterile capsule, and make cultures and cover-slide preparations from each.

15. Examine the animal thoroughly for any pathological lesions.

16. Finally, plunge the whole animal with the board into a pail of lysol kept for the purpose, and cremate as soon as possible.

17. Allow the films to dry in the air, and stain by appropriate methods according to organism present.

18. Incubate the cultures at 37° C. and examine daily for six days.

The foregoing is a brief outline of the main principles to be observed in making a post-mortem in the bacteriological laboratory.

It is abbreviated from Eyre's "Bacteriological Technique," and, with modifications, can be used for any animal, bird, etc., used in the laboratory for the purpose of inoculation.

In such a small volume as the present it is, of course, quite impossible to describe the various post-mortem lesions to be sought, and for a full description the student is referred to standard works on bacteriology. But the following are a few brief notes on the chief lesions to be looked for after inoculation with some of the more important and best-known pathogenic bacteria :

Tubercle Bacillus.—Animals can be inoculated and infected with this bacillus in any of the following ways :

By injection of the bacillus into the subcutaneous tissues (usual method for milk, etc.), the peritonem, anterior chamber of the eye, veins, by feeding with

cultures, and by the inhalation by the animal of a spray preparation of the bacillus.

NOTE.—In the infected animal inoculated subcutaneously a large swelling occurs at the site of inoculation. About the tenth day this becomes soft and caseous; lymphatic glands become enlarged in regular sequence from two to three weeks; there is gradual loss of weight, and death usually about the tenth week. Tuberculous nodules present in spleen, liver, and lungs.

The guinea-pig is especially susceptible to the tubercle bacillus.

Anthrax.—Usually fatal within two days. Note the swollen, gelatinous mass at site of inoculation (stain portion to demonstrate presence of the bacillus). Internal organs, especially spleen, show congestion and cloudy swelling, with capillaries crowded with the bacillus.

Glanders.—The mallein reaction and Strauss's reaction have already been mentioned under *B. mallei*. A guinea-pig is used to demonstrate the reaction.

Diphtheria.—Usually death within thirty-six hours. Look for a small patch of greyish membrane at site of inoculation, with inflammatory œdema of surrounding tissues. Bacillus remains practically local. There may be a membrane on any of the mucous surfaces.

Pneumococcus.—Death usually in twenty-four to forty-eight hours; general septicæmia; spleen specially enlarged and firm. Blood cultivations show presence of encapsuled diplococcus. The mouse is especially susceptible.

Pneumobacillus.—The lesions post-mortem are much the same as above, but the organism is stated not to be so virulent.

Streptococcus Pyogenes.—The lesions of this organism vary enormously according to its virulence, from a mild local œdema only to a virulent and rapidly fatal septicæmia.

Staphylococcus Aureus.—Usually the lesion observed is an acute local inflammation followed by suppuration.

Tetanus Bacillus.—The tetanic spasms observed in the animal before death are the main symptoms. Death usually occurs from two to five days after inoculation, and very little is found post-mortem. The bacillus remains local at the site of inoculation.

Trypanosomiasis.—After inoculation, the animal (usually a rat) shows very few symptoms up till a few hours before death. The blood is found to be crowded with the trypanosomes.

Plague.—Post-mortem examinations of plague-infected rats in a well-marked case may show the lesions as follows: Enlarged congested spleen, with occasionally multiple small areas of necrosis, together with marked swelling and congestion of the inguinal and axillary lymphatic glands. Smears taken from the spleen will show large numbers of a Gram-negative, bipolar bacillus. But it must be borne in mind that the spleens of healthy rats vary enormously, and a rat heavily infected with the plague bacillus may show very little enlargement of the spleen. The only really reliable test is a bacteriological examination of spleen smears.

Spirochæta Pallida.

This organism is extremely minute and difficult to find, even on a slide which contains large numbers. It is a spiral-shaped organism, and should show at least from six to eight curves, though longer forms are occasionally seen. It measures from 4 to 14 μ in length (roughly, a little more than twice the diameter of a red blood-corpuscle), and is 0.25 μ thick. In fresh specimens it exhibits movements of three kinds: rotation on its long axis, gliding movements, and movements of flexion of the whole body. It is stated to possess a single delicate flagellum at each pole (Schaudinn).

To detect the presence of the spirochæte, a quick and easy method is to scrape the surface of a hard chancre, preferably with a broad-bladed needle, such as is used for puncturing the lobe of the ear when taking blood for a blood-count. Transfer each scraping obtained to a solution of 0.7 per cent. sodium chloride solution in a very small test-tube (similar to those used in preparing the serum dilutions in Widal's reaction). Having in this manner obtained a thick emulsion, prepare a hanging-drop specimen, and examine microscopically for movements, closing down the Iris diaphragm so that the field is not too bright. Take several smear preparations from the original scraped chancre by pressing a glass-slide directly on to the scarified surface, allow to dry in the air, fix with alcohol, and stain directly by Giemsa's method, as described under Staining Methods.

Small quantities of the emulsion in saline solution

can also be spread evenly over glass-slides, allowed to dry in air, and stained with Giemsa's stain.

To demonstrate this organism in tissues, the silver pyrogallate method as described in the chapter on Staining Methods is recommended.

Spirochæta refringens can be distinguished from the *S. pallida* by being more highly refractile. It is also longer, thicker, and has fewer and wider curves, which are very irregular.

Trypanosomata.

Of the many varieties of this protozoon, the one best known and most commonly met with is the *Trypanosoma Lewisi*, which was observed in 1878, infecting the blood of rats.

To demonstrate the presence of this organism in the blood of a rat, a small quantity of the blood (about 1 drop) is allowed to fall on a glass-slide. By means of another slide this is spread evenly to form a film, which is allowed to dry in the air, and finally stained by Leishman's method; or simply staining the fixed film with a weak solution of carbol-fuchsin answers very well, but does not so effectively bring out the chromatin structures. To examine the living specimens, it is simply necessary to allow a drop of the fresh blood to spread itself out beneath a cover-glass.

For staining Trypanosomata in sections the process is rather long and complicated, that of Leishman giving very good results. (For details, see Muir and Ritchie's "Bacteriology," p. 545.) The essential part of the method is the application of fresh blood to the

section before staining, though what effect this has is not known.

The organism resembles a fusiform mass of protoplasm, which at one end passes into a pointed flagellum ; it varies in size, but is usually about $30\ \mu$ long and from $1.5\ \mu$ to $3\ \mu$ broad. In fresh specimens it is actively motile, with an undulatory movement of its protoplasm and a lashing of the flagellum.

In stained specimens two chromatin-like bodies are usually present, staining purple—one near the middle, and the other near the anti-flagellar end. A vacuole can also often be seen to exist, and an undulating membrane can sometimes be demonstrated.

Intermediate host of human trypanosome (*T. Gambiense*) is the Tsetse fly (*Glossina palpalis*).

Detection of the Malaria Parasite.

To appreciate the principles on which the blood examination of a malarial patient is conducted, it must be borne in mind that the parasite is intracorpuscular. Therefore, for the detection it is necessary that the red blood-corpuscles in which it is contained should lie flat on the slide in a single layer, and present their surfaces, not their edges, to the observer. The portion of the slide described by Manson as the "single-layer zone" is the best for a beginner to examine, for in this zone the corpuscles all lie flat with their edges approximated, but not overlapping.

The best case for examination, if practicable, is one of quartan infection, and it is necessary to examine the patient's blood just before or at the time of a rigor. During this period, in quartan and

benign tertian infections, there should be little difficulty in discovering large parasites, also the abundance of coarse pigment they contain.

Failing the above, a long-standing case of recurring malaria, with marked cachexia, will afford the best opportunity, as in this form it is generally an easy matter to find crescents and spheres of considerable size, possessing abundance of pigment and a very definite and striking shape.

The intracorpuseular forms most frequently met with have the appearance either of small specks of pale protoplasm or of larger masses of pale protoplasm containing black grains of pigment.

Close observation with a warm stage and fresh specimens will demonstrate in the former the presence of amœboid movement.

The space occupied by these intracorpuseular pigmented parasites varies from a very small portion to nearly the whole area of the infected corpuscle.

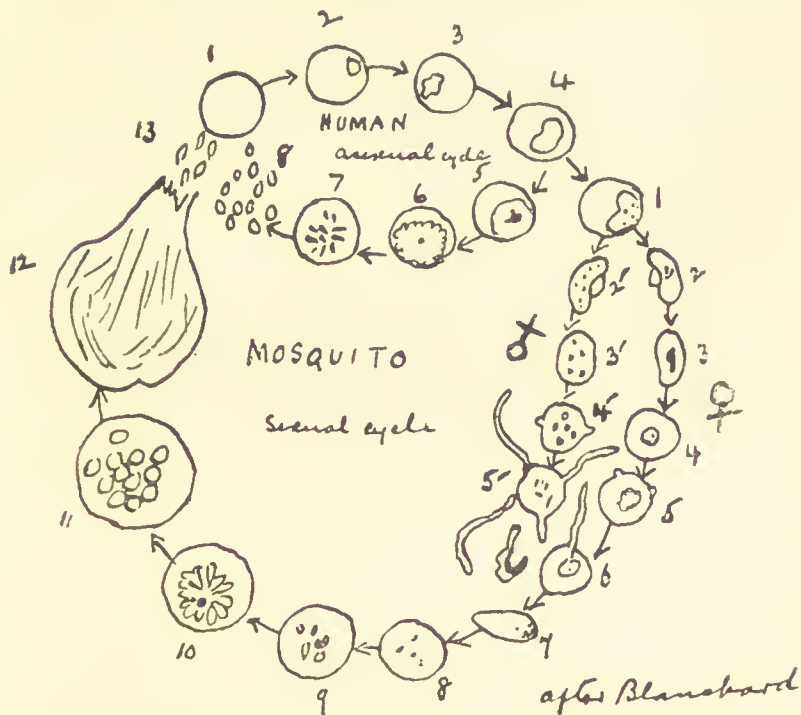
It is important that vacuoles should not be mistaken for the parasites; they are distinguished by being clear, well-defined, with sharp edges, and show no amœboid movements, nor do they stain.

Intracorpuseular malaria parasites, on the contrary, are dim and, as a rule, ill-defined, having soft, shaded-off edges, carrying pigment grains, and showing amœboid movements; they also take the appropriate stains.

Flagellated bodies are seldom encountered in the ordinary quartan and tertian forms of malaria, but are fairly frequent in malignant crescent infection. The best time to find them in such cases is said to be

during the hot stage of the fever. With patience the gradual evolution of the flagellated body from crescent through oval and sphere can be followed.

Polymorphonuclear leucocytes may sometimes be observed to contain pigment granules, set free by



the falling to pieces of the red corpuscles containing the segmented parasites.

According to Metschnikoff, the lymphocyte has no phagocytic action in malaria, and this observation is confirmed by Manson.

A detailed description of the many forms of the parasite is quite impossible in a volume of this size and scope, but the writer ventures to hope that the

diagram on p. 71, modified from Blanchard, may be of assistance.

Human Cycle :

1. Normal red blood-cell.
- 2 to 5. Red cells containing amœbulæ.
6. Roseate arrangement.
7. Sporocytes ready for discharge.
8. Escaped sporocytes.

Mosquito Cycle :

1. Young gametocyte.
- 2, 3, 4, 5. Macrogametocytes, or female gametes.
- 2', 3', 4', 5'. Microgametocytes, or male gametes.
6. Microgamete piercing female macrogamete.
7. Travelling vermicle.
8. Young zygote.
- 9, 10. Zygotomeres.
11. Zygotomeres.
12. Mature zygote.
13. Liberated sporozoites.

The examination of stained specimens of malarial blood is best conducted with a $\frac{1}{12}$ -inch oil-immersion lens, an eyepiece of rather low power, a substage condenser, and a good, but not too bright, illumination ; also, several slides should be thoroughly examined before a negative decision is given.

To demonstrate amœboid movements in fresh blood specimens, the $\frac{1}{6}$ -inch objective can be used, with the condenser racked down.

NOTE.—It is useless to examine blood for the

intracorporeal forms of the malarial parasite after full doses of quinine have been given, as the crescent form alone is unaffected by drugs.

Intermediate host = mosquito.

PREPARATION OF FLUID BLOOD-FILMS.

1. Cleanse one of the patient's finger-tips with alcohol, and dry it.

2. Prick the part with a sterile needle.

3. Wipe away the first drop of blood that exudes.

4. Gently squeeze the finger-tip to cause another small drop of blood to exude.

5. Touch the droplet lightly with the centre of a cover-glass. (Take care that the latter does not come in contact with the skin.)

6. Immediately drop the cover-glass (blood side downwards) on to a glass-slide.

7. The film is now ready for examination.

NOTE.—No pressure whatever must be used to cause the blood to spread out. If glasses and skin are quite clean, the blood will at once run out in a very fine film. If a prolonged examination is required, the preparations should be ringed with vaseline to prevent evaporation.

Examination of Blood for Filarial Embryos.

There are, broadly speaking, four varieties of filarial embryos present in human blood, their habits and microscopical characters being roughly as follows :

<i>Variety.</i>	<i>Sheath.</i>	<i>Shape of Tail.</i>	<i>Periodicity.</i>
<i>F. nocturna</i> (Bancrofti)	Present	Sharply pointed	Nocturnal in peripheral blood
<i>F. diurna</i> (Loa)	Present	Sharply pointed	Diurnal in peripheral blood
<i>F. perstans</i>	Absent	Blunt, truncated	None
<i>F. Demarquayi</i>	Absent	Sharply pointed	None

It is necessary for the student to bear these facts in mind when proposing to draw blood from a patient to establish the diagnosis of filariasis.

Intermediate host is the mosquito.

Direct Examination of Fresh Blood.—All that is necessary is to allow one drop of blood to fall on a glass-slide; place a cover-glass over the drop of blood, and examine wet by means of the $\frac{1}{8}$ -inch objective.

The filarial embryos can be observed quite easily, and are very active. They are usually about 0·3 millimetre in length, and about 00·07 millimetre in thickness.

To Stain and Mount Filaria Embryos.—Allow about three drops of blood to fall on the slide; run them together in a thick film.

Dry in air.

Place in distilled water to take out hæmoglobin.

Dry in air.

Stain with hæmatoxylin (warming if necessary).

Wash well in water.

Dry.

It is unnecessary to mount the films prepared as above if the slides are to be used at once. If required for permanent specimens, they can be mounted with Canada balsam and a cover-slip in the usual way.

Leishman-Donovan Bodies.

These bodies are best observed in smears prepared from a liver puncture taken from a patient suffering from Kala-azar.

They are best seen in thin smears stained as described for Leishman's stain.

The Leishman-Donovan bodies may be free in the liver smear, but are more frequently seen within the large mononuclear leucocytes.

They are oval in shape, about $2.5\ \mu$ to $3.5\ \mu$ in diameter. The protoplasm usually stains a bluish colour; within this protoplasm can be seen two bodies taking on the bright red colour of nuclear matter. The larger red body stains less intensely than the smaller, and is usually round or oval in shape. The smaller red body is rod-shaped, and stains a deep red colour.

Similar bodies to the above have been found in the disease known as "Delhi boil."

These bodies can be cultivated in citrated blood media.

Intermediate host : probably bed-bug (*Cimex*).

Spirillum or Spirochæta Obermeieri (Spirillum of Relapsing Fever).

In blood-films stained by Leishman's method these organisms appear as long spiral filaments (about three times as long as a red blood-corpuscle); their extremities are finely pointed. They are actively motile in fresh blood specimens, and are very numerous in blood specimens taken during the fever, but begin to disappear shortly before the crisis, and after the crisis they are usually entirely absent from the circulating blood. All attempts at cultivation have so far failed, but they can be kept alive in citrated blood outside the body several days.

Intermediate Host.—*S. Obermeieri*: probably louse (*Pediculus*) or bugs (*Cimex*). *S. Duttoni*: ticks (*Ornithodoros*).

Piroplasma.

This blood-parasite is rarely met with by the ordinary medical student, but is common in veterinary practice.

Thin blood-films are prepared and stained with either Leishman's or Giemsa's stain.

The parasite is seen to be inside the red corpuscles; it is usually shaped like two clubs joined together by their narrow ends. The chromatin portion stains red, and the protoplasmic portion blue, by either of the above stains.

Intermediate host: the tick.

Amœbæ.

The two common varieties are as follows: *Entamœba coli* and *Entamœba tetragena*. Their differentiation is far from easy, and even amongst experts is still a debated point; therefore, as it is in connection with dysenteric stools that the student or practitioner will be asked to examine fæces for the presence of amœbæ, a short description of *Entamœba tetragena* only is given. The most satisfactory method of examining either fæces or pus from a liver abscess is to simply take a small portion of material on a glass-slide, mix gently with saline solution, cover with a thin cover-slip, and examine at once with the $\frac{1}{6}$ -inch objective, using the diaphragm to shut off a large portion of the transmitted light. Some observers use stains of various descriptions, but the writer has always obtained good results as above; the essential thing is to obtain a fresh specimen, as amœbæ rapidly die in putrefying fæces.

Entamœba tetragena as seen in dysenteric stools occurs in the form of rounded, oval, or pear-shaped bodies, measuring from $12\ \mu$ to $50\ \mu$ in diameter. If carefully observed, a clear, highly refractile ectoplasm and a granular endoplasm can be made out. A nucleus can be observed with difficulty, usually eccentric in position. Amœboid movements and the protrusion and retraction of pseudopodia can be observed.

Amœbæ can be cultivated outside the human body on plain agar (alkaline reaction) in association with the *Bacillus coli communis*.

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